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REMARKS

Specification Amendments

The Specification is amended to add low, medium and high stringency conditions. Support for this amendment is found, for example, in the Specification (at page 26, lines 25-28), which states "See, e.g., Ausubel *et al.*, eds. *Current Protocols in Molecular Biology*, Wiley Interscience, N.Y. (1987, 1992, 1993), and Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989), the entire contents of which are incorporated herein by reference" and in Ausubel *et al.*, *supra*, at 2.10.2-2.10.3. The relevant portions of Ausubel *et al.* and Sambrook *et al.* are attached hereto as Exhibits A and B, respectively. No new matter has been added.

Claims

A complete Claim Listing is submitted in this Amendment as required by the Examiner in the Office Communication dated January 2, 2004. No claims have been amended in this Amendment. Claims 3, 15 and 20 were previously canceled in the Amendment dated September 30, 2002.

Rejection of Disclosure

According to the Examiner, the instant specification, which claims priority to US Application No. 09/133,119, now U.S. Patent No. 6,277,969, as a continuation, differs from the '119 specification at page 29, line 26, which has deleted the definitions for low, medium and high stringency that are present in the '119 application. Thus, the Examiner states that the instant application is a continuation-in-part of the '119 application rather than a continuation.

Applicants respectfully disagree. The '119 application, as originally filed, did not contain the definitions for low, medium and high stringency. These definitions were added to the specification in an Amendment that was filed with the United States Patent and Trademark Office (USPTO) on March 22, 2000, in response to an Office Action mailed from the USPTO on September 22, 1999. The Patent Office entered the amendment into the record. Therefore, the disclosure of the instant application, as originally filed, is substantially the same as that of the parent '119 application as originally filed. Thus, the instant application is properly designated as

a continuation of the '119 application. Reconsideration and withdrawal of the objection are respectfully requested.

The Examiner also objected to the specification for failing to provide proper antecedent basis for the claimed subject matter, specifically the phrase "high stringency" is missing from the specification.

Applicants have amended the instant specification to include and define low, medium and high stringency. Again, this amendment was acceptable to the Patent Office and entered into the record in prior application 09/133,119 (now U.S. Patent No. 6,277,969), which is a parent application to the instant application. Furthermore, such hybridization conditions are well known to those of skill in the art. For example, stringent conditions are described in Sambrook, J. *et al.*, *supra,* at page 11.45 -11.61 ("Conditions for Hybridization of Oligonucleotide Probes") and Ausubel, F.N. *et al., Current Protocols in Molecular Biology*, at Chapter 2 ("Preparation and Analysis of DNA"), Chapter 14 ("In situ Hybridization and Immunochemistry") and Chapter 15 ("The Polymerase Chain Reaction"), Greene Publishing Assoc. and Wiley-Interscience (1989). Relevant portions of Chapter 2 of Ausubel *et al.* are included herein as Exhibit A. Relevant portions of Sambrook are included herein as Exhibit B. Hybridization conditions listed in these references include ionic strength and temperature of the post-hybridization wash. See, for example, Exhibit A at 2.10.2-3 and 2.10.10-11 and Exhibit B at 11.45. One skilled in the art would be able to optimize hybridization conditions by varying the specifically exemplified conditions discussed in the references and Specification. Such optimization is routine in the art.

Reconsideration and withdrawal of the objection are respectfully requested.

Rejection of Claims 7-12 Under 35 U.S.C. §101

Claims 7-12 are rejected under 35 U.S.C. §101 as directed to non-statutory subject matter. According to the Examiner, Claims 7-12 are drawn to expression vectors comprising a nucleic acid sequence and can be construed as reading on an expression vector comprised within a human having undergone treatment comprising the administration of said claimed expression vectors.

Applicants are confused by the Examiner's rejection and clarification is respectfully requested. Claims 7-12 are directed towards expression vectors comprising the nucleic acid molecule according to Claims 1-6, respectively. 35 U.S.C. §101 states:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

The claimed expression vectors comprising nucleic acid molecules are a new and useful composition. The claimed expression vectors are a nonnaturally occuring composition of matter and are clearly new and patentable (see MPEP §2105). Additionally, the claimed expression vectors are useful because, as discussed below, the specification discloses many uses for the claimed expression vectors. Furthermore, the instant claims are representative of standard claims presented to, and issued by, the USPTO. For example, see Claims 3 and 4 of U.S. Patent No. 6,277,969, which the Examiner has allowed to issue (a copy of the cover page and claims for 6,277,969 is enclosed as Exhibit C for the Examiner's convenience). See also Claim 13 of U.S. Patent No. 6,573,077; Claim 8 of U.S. Patent No. 6,573,368; Claims 17 and 18 of U.S. Patent No. 6,569,667; and Claim 5 of U.S. Patent No. 6,570,062 (copies of the cover page and claims for the referenced patents are enclosed as Exhibits D-G for the Examiner's convenience).

MPEP §2107.02 states:

> Office personnel should also be especially careful not to read into a claim unclaimed results, limitations or embodiments of an invention. See *Carl Zeiss Stiftung v. Renishaw PLC*, 945 F.2d 1173, 20 USPQ2d 1094 (Fed. Cir. 1991); *In re Krimmel*, 292 F.2d 948, 130 USPQ 215 (CCPA 1961). Doing so can inappropriately change the relationship of an asserted utility to the claimed invention and raise issues not relevant to examination of that claim.

The Examiner appears to be reading an unclaimed embodiment into the Claims 7-12. The instant claims are novel composition claims and do not recite methods of delivery for gene therapy. Thus, the Examiner has changed the relationship of an asserted utility to the claimed invention and has raised an issue not relevant to the examination of Claims 7-12.

As Claims 7-12 are directed to patentable subject matter under 35 U.S.C. §101, the amendment suggested by the Examiner need not be addressed. Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 7-12 Under 35 U.S.C. §112, First Paragraph

Claims 7-12 are rejected under 35 U.S.C. §112, first paragraph, because, according to the Examiner, the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. The Examiner has rejected "the claims on the grounds that the claims are clearly intended to encompass methods of gene therapy." However, according to the Examiner, the specification is not enabling for gene therapy.

Again, Applicants are confused and further clarification of this rejection is respectfully requested. The claims are not drawn to or limited by any recited method or use. Claims 7-12 are directed towards expression vectors comprising the nucleic acid molecule according to Claims 1-6, respectively. The specification provides ample teachings to enable one of skill in the art to clone an isolated nucleic acid molecule according to Claims 1-6 into an expression vector (see Detailed Description at page 44, lines 17 to 25).

MPEP 2164.01(c) states:

> when a compound or composition claim is not limited by a recited use, any enabled use that would reasonably correlate with the entire scope of that claim is sufficient to preclude a rejection for nonenablement based on how to use.

The specification enables many uses for the claimed expression vectors. For example, on page 30, line 5 to page 31, line 2, the specification describes that a polynucleotide encoding an anti-TNF variable or constant region can be cloned into an expression vector which is capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2. The instant claims are representative of standard claims presented to, and issued by, the USPTO. Moreover, instant Claims 7-12 are directed to expression vectors with a similar scope of coverage as Claims 3 and 4 of the parent application U.S. Patent No. 6,277,969, which the Examiner found to be enabled by the same specification. Therefore the instant specification

enables one skilled in the art to make and use the invention commensurate in scope with Claims 7-12. Reconsideration and withdrawal of the rejection are respectfully requested.

Information Disclosure Statement

A Supplemental Information Disclosure Statement (SIDS), form PTO-1449 (1 sheet listing references AF2-AJ2 and AP7) and fee of $180 was filed on July 29, 2003, with the Amendment that the Examiner found to be not fully responsive, and was received by the U.S. Patent and Trademark Office on July 31, 2003, as evidenced by the copy of the date-stamped postcard attached herewith. Copies of the SIDS and form PTO-1449 are also attached. Entry of the SIDS filed on July 29, 2003 is respectfully requested.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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different amounts of hybrid .e DNA; if this occurs, the relative intensities displayed by two dots after hybridization will not be representative of the amount of target DNA that each contains.

The protocols for blotting uncharged nylon and nitrocellulose membranes attempt to ensure complete denaturation through the use of two denaturation steps—a heat denaturation before application to the membrane and an alkaline denaturation after application. Heat denaturation on its own is rarely adequate, as the DNA can renature fairly extensively before application to the membrane, even if plunged into ice on removal from the incubator. Blotting, whether manual or with a manifold, takes time, with some samples being blotted more quickly than others, so differential renaturation is a possibility. The second denaturation step, when the membrane is placed on a filter paper soaked in alkali, is intended to bring all the DNA back to an equal standing. Note that these problems do not arise with alkaline blotting onto positively charged nylon, as the high pH of the blotting solution maintains the DNA in a denatured state. Alkaline blotting is therefore the method of choice for DNA dot and slot blots where comparisons between different samples are to be made.

A second variable results from the purity of the DNA samples. With a Southern transfer, the gel electrophoresis step helps to fractionate away impurities, so the DNA that is transferred is relatively clean. Dot/slot blotting with bulk DNA lacks the benefit of a gel fractionation step, and the resulting co-blotted impurities can have unpredictable effects on hybridization, possibly reducing signal by blocking access to the hybridization sites, or increasing signal by trapping the probe. This must be taken into account if the signal intensity is to be used to estimate the absolute amount of target DNA, through comparison with a control dilution series. Copy number reconstruction by dot blot analysis is particularly suspect, as comparison between blots of cellular and plasmid DNA are reliable only if both types of DNA are scrupulously purified.

Troubleshooting

As with Southern blotting (*UNIT 2.9A*), most problems with dot and slot blots become apparent only after hybridization . ysis. The warning signs detailed in the commentary to *UNIT 2.9A* also hold for dot/slot blotting; other problems are described in *UNIT 2.10* (see Table 2.10.4 for troubleshooting).

Anticipated Results

The procedures yield a clear white membrane carrying applied DNA in amounts up the carrying capacity of the matrix (Table 2.9.1, *UNIT 2.9A*). No data is generated until the membrane is subjected to autoradiography; anticipated results of autoradiography are discussed in *UNIT 2.10*.

Time Considerations

A manifold or manual blot can be set up and ready for sample application in as little as 15 min. After sample application, it takes about 3 hr to complete the protocol with a nitrocellulose membrane (most of this being the baking step), 60 min with an uncharged nylon membrane, and 30 min with a positively charged nylon membrane. The rate-determining step is sample application. Manifold application of clean samples (where no blockages occur) takes 5 min, but application by hand can take several hours if the sample volume is large and multiple additions have to made.

Literature Cited

Kafatos, F.C., Jones, C.W., and Efstratiadis, A. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucl. Acids Res.* 7:1541-1552.

Key Reference

Dyson, N.J. 1991. Immobilization of nucleic acids and hybridization analysis. *In* Essential Molecular Biology: A Practical Approach, Vol. 2 (T.A.Brown, ed.) pp. 111-156. IRL Press at Oxford University Press, Oxford.

Describes dot and slot blotting in some detail.

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Hybridization Analysis of DNA Blots

The principle of hybridization analysis is that a single-stranded DNA or RNA molecule of defined sequence (the "probe") can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"), with the stability of the hybrid depending on the extent of base pairing that occurs. Experimentally, the analysis is usually carried out with a probe that has been labeled and target DNA that has been immobilized on a membrane support. Hybridization analysis is sensitive and permits detection of single-copy genes in complex genomes. The technique has widespread applications in molecular biology.

The first stage in a hybridization experiment is to immobilize the denatured nucleic acids on a suitable solid support. Methods for achieving this with gel-fractionated and bulk DNA are described in *UNITS 2.9A & 2.9B*. The labeled probe is then applied in a solution that promotes hybridization. After a suitable incubation, the membrane is washed so that any nonspecifically bound probe is removed, leaving only probe that is base-paired to the target DNA. By controlling the stringency of the washing conditions, decisions can be made about whether to target sequences that are 100% complementary to the probe, or allow some mismatching so that sequences with lower degrees of similarity are also detected. The latter approach (heterologous probing) is used to study related sequences in a single or more than one genome.

Hybridization analysis was originally carried out with long (100 to 1000 bp), radioactively labeled DNA probes. Other types of probe (RNA, oligonucleotide) have more recently been introduced, as have nonradioactive labeling and detection strategies. In addition, improvements in understanding of the factors that influence hybrid stability and hybridization rate have led to a proliferation of reagents and protocols for hybridization analysis. Finding one's way through the maze can be a daunting task, especially as protocols that work well with one probe-target combination may not work so well if either member of the partnership is changed. The approach taken here is to present as the basic protocol an unsophisticated procedure for hybridization analysis with a radiolabeled DNA probe. Despite its lack of embellishments, the protocol gives acceptable results with Southern and dot blots on nitrocellulose and nylon (uncharged and charged) membranes. The alternate protocol describes a similar method for probing DNA blots with a radiolabeled RNA probe. A support protocol for stripping blots to ready them for reprobing is also provided.

Relevant units elsewhere in the manual include the following: *UNITS 3.18 & 3.19* describe the preparation of nonradioactive probes and their use in hybridization analysis; *UNIT 4.9* covers hybridization analysis of immobilized RNA; *UNIT 6.3* describes hybridization analysis of recombinant clone libraries; and *UNIT 6.4* explains how to use labeled oligonucleotides as hybridization probes.

These hybridization protocols should not be read in isolation. The commentary describes various modifications that can be introduced, including changes to prehybridization, hybridization, and wash solution formulations, and alterations to incubation times and conditions, the latter including a discussion of the wash conditions compatible with different degrees of stringency. The intention is provide the reader with sufficient data to make well-informed decisions about how to modify the basic and alternate protocols for specific applications.

CAUTION: Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves and their clothing. When working with ^{32}P, investigators should frequently check themselves and the working area for

radioactivity using a hand-held monitor. Any radioactive contamination should be cleaned up using appropriate procedures. Radioactive waste should be placed in appropriately designated areas for disposal. Follow the guidelines provided by your local radiation safety adviser.

HYBRIDIZATION ANALYSIS OF A DNA BLOT WITH A RADIOLABELED DNA PROBE

BASIC PROTOCOL

This protocol is suitable for hybridization analysis of Southern transfers (*UNIT 2.9A*) and dot and slot blots (*UNIT 2.9B*) with a radioactively labeled DNA probe 100 to 1000 bp in length. The steps employ nylon membranes (uncharged or positively charged) but are suitable for nitrocellulose if modified as described in the annotations. The commentary describes how to tailor the protocol for individual requirements.

A hybridization experiment can be divided into three stages. First, the membrane is incubated in a prehybridization solution containing reagents that block nonspecific DNA binding sites on its surface, thereby reducing background hybridization. In this protocol, the blocking agents are Denhardt solution and denatured salmon sperm DNA; alternatives are described in the commentary. In the second stage, the prehybridization solution is replaced by fresh buffer containing the labeled probe, and an overnight incubation is carried out to allow the probe to bind to target sequences in the immobilized DNA. During this hybridization step, the probe pairs not only with target sites that have 100% complementarity with the probe, but also with related sequences. In the final stage of the experiment the membrane is washed with a series of solutions that gradually remove bound probe molecules until only highly matched hybrids remain.

Materials

DNA to be used as probe
Aqueous prehybridization/hybridization (APH) solution, room temperature and 68°C
2× SSC/0.1% (w/v) SDS
0.2× SSC/0.1% (w/v) SDS, room temperature and 42°C
0.1× SSC/0.1% (w/v) SDS, 68°C
2× and 6× SSC (*APPENDIX 2*)
Hybridization oven (e.g., Hybridiser HB-1, Techne) *or* 68°C water bath or incubator
Hybridization tube *or* sealable bag and heat sealer
Additional reagents and equipment for DNA labeling by nick translation or random oligonucleotide priming (*UNIT 3.5*), measuring the specific activity of labeled DNA and separating unincorporated nucleotides from labeled DNA (*UNIT 3.4*), and autoradiography (*APPENDIX 3*)

1. Label the probe DNA to a specific activity of $>1 \times 10^8$ dpm/μg by nick translation or random oligonucleotide priming. Measure the specific activity and remove unincorporated nucleotides.

   The probe should be a double-stranded DNA fragment, ideally 100 to 1000 bp in length. Usually the probe DNA is obtained as a cloned fragment (Chapter 1) which is purified from the vector by restriction digestion (UNIT 3.1) followed by recovery from an agarose gel (UNIT 2.6).

2. Wet the membrane carrying the immobilized DNA in 6× SSC.

   The membrane is blotted as described in UNIT 2.9A. Do not handle the membrane: use clean blunt-ended forceps.

3. Place the membrane, DNA-side-up, in a hybridization tube and add ~1 ml APH solution per 10 cm^2 of membrane.

   Prehybridization and hybridization are usually carried out in glass tubes in a commercial hybridization oven. Alternatively, a heat-sealable polyethylene bag can be used. The membrane should be placed in the bag, all edges sealed using a heat sealer, and a corner cut off. The APH solution is then pipetted into the bag through the cut corner and resealed.

4. Place the tube in the hybridization oven and incubate 3 hr with rotation at 68°C.

   If using a bag, shake slowly in a suitable incubator or water bath.

   If using a nylon membrane, reduce the prehybridization period to 15 min, but warm the prehybridization/hybridization solution to 68°C before adding to the membrane.

5. Denature the probe DNA by heating for 10 min in a water bath or incubator at 100°C. Place in ice.

   Step 5 should be done immediately before step 6, with a minimum delay between them.

6. Pour the APH solution from the hybridization tube and replace with an equal volume of prewarmed (68°C) APH solution. Add denatured probe and incubate with rotation overnight at 68°C.

   The probe concentration in the hybridization solution should be 10 ng/ml if the specific activity is 10^8 dpm/μg, or 2 ng/ml if the specific activity is 1×10^9 dpm/μg. If using a bag, cut off a corner, pour out the prehybridization solution, add the hybridization solution plus probe, and reseal. It is very difficult to avoid contaminating the bag sealer with radioactivity; furthermore, the sealing element (the part that gets contaminated) is often difficult to clean. Hybridization bags are therefore not recommended.

7. Pour out the APH solution, using the appropriate disposal method for radioactive waste, and add an equal volume of 2× SSC/0.1% SDS. Incubate with rotation for 10 min at room temperature, changing the wash solution after 5 min.

   CAUTION: *All wash solutions must be treated as radioactive waste and disposed of appropriately.*

   To reduce background, it may be beneficial to increase the volume of the wash solutions by 100%. If using a bag, transfer the membrane to a plastic box for the washes.

8. Replace the wash solution with an equal volume of 0.2× SSC/0.1% SDS and incubate with rotation 10 min at room temperature, changing the wash solution after 5 min (this is a low-stringency wash; see commentary).

9. If desired, carry out two further washes as described in step 8 using prewarmed (42°C) 0.2× SSC/0.1% SDS for 15 min each at 42°C (moderate-stringency wash).

10. If desired, carry out two further washes using prewarmed (68°C) 0.1× SSC/0.1% SDS for 15 min each at 68°C (high-stringency wash).

11. Pour off the final wash solution, rinse the membrane in 2× SSC at room temperature, and blot excess liquid. Wrap in plastic wrap.

    Do not allow the membrane to dry out if it is to be reprobed.

12. Set up an autoradiograph (*APPENDIX 3*).

HYBRIDIZATION ANALYSIS OF A DNA BLOT WITH A RADIOLABELED RNA PROBE

ALTERNATE PROTOCOL

Purified RNA polymerases from bacteriophages such as SP6, T3, and T7 (*UNIT 3.8*) are very efficient at synthesizing RNA in vitro from DNA sequences cloned downstream of the appropriate phage promoter (Little and Jackson, 1987). Several micrograms of RNA can be obtained from 1 μg of DNA template in a 10-min reaction. If a radiolabeled ribonucleotide is added to the reaction mixture, the polymerase synthesizes uniformly labeled RNA with specific activities up to and beyond 10^9 dpm/μg. The fact that RNA probes are single-stranded gives them certain advantages over DNA probes. RNA probes do not need to be denatured before being added to the hybridization solution, and remain fully available for hybridization during the course of the incubation. In contrast, the "potency" of a double-stranded DNA probe gradually declines as the complementary strands of the probe reanneal during the hybridization reaction, reducing the amount of probe available to hybridize to the target.

The hybridization protocol for an RNA probe is not greatly different from that with labeled DNA. Formamide is usually included in the prehybridization/hybridization solutions, because in the presence of formamide an RNA-DNA hybrid is more stable than the equivalent DNA-DNA hybrid. Carrying out the hybridization with formamide also permits the incubation to be at a lower temperature without loss of stringency. Single-strand-specific RNases are added to one or more of the wash solutions to remove nonhybridized probe molecules, considerably reducing background.

The protocol includes details of probe preparation (Mundy et al., 1991). The hybridization procedure is suitable for both nitrocellulose and nylon membranes, though backgrounds may be higher with nylon.

Additional Materials

TE buffer, pH 8.0 (*APPENDIX 2*)
Labeling buffer
Nucleotide mix (see reagents and solutions)
200 mM dithiothreitol (DTT), freshly prepared
20 U/μl human placental ribonuclease inhibitor
[α-^{32}P]UTP: 20 mCi/ml (800 Ci/mmol) or 10 mCi/ml (400 Ci/mmol)
SP6 or T7 RNA polymerase (*UNIT 3.8*)
RNase-free DNase I (*UNIT 3.12*)
0.25 M EDTA, pH 8.0 (*APPENDIX 2*)
Formamide prehybridization/hybridization (FPH) solution
2× SSC (*APPENDIX 2*) containing 25 μg/ml RNase A + 10 U/ml RNase T1 (*UNIT 3.13*)
Additional reagents and equipment for cloning and purifying plasmid DNA (Chapter 1), phenol extraction and ethanol precipitation (*UNIT 2.1*), restriction digestion of DNA (*UNIT 3.1*), measuring the specific activity of and separating unincorporated nucleotides from labeled RNA (*UNIT 3.4*), and autoradiography (*APPENDIX 3*)

Prepare the RNA probe

1. Clone into a suitable vector (Table 2.10.1) the DNA fragment that will be transcribed into the RNA probe.

   DNA must be of high purity, so use a method that includes a CsCl/ethidium bromide equilibrium centrifugation step (UNIT 1.7).

2. Linearize the DNA by restriction digestion immediately downstream of the cloned fragment.

Linearization introduces an endpoint for RNA synthesis. This ensures that enzymes and substrates are not wasted by transcribing downstream vector DNA, and also increases the specificity of the probe by excluding unwanted sequences.

3. Purify the DNA from the restriction enzyme by phenol extraction and ethanol precipitation. Resuspend in TE buffer, pH 8.0, at a concentration of 1 mg/ml.

4. Mix the following at room temperature:

   4 µl labeling buffer
   1.5 µl nucleotide mix
   1 µl 200 mM DTT
   1 µl (20 U) human placental ribonuclease inhibitor
   2 µg purified plasmid DNA from step 3
   100 to 200 µCi [α-^{32}P]UTP
   H_2O to a final volume of 20 µl.

   These are mixed at room temperature, as the spermidine in the labeling buffer may precipitate on ice.

5. Add 5 U SP6 or T7 RNA polymerase. Incubate for 1 hr at 40°C for SP6 or at 37°C for T7.

6. Add 2 U RNase-free DNase I and incubate at 10 min 37°C. Stop the reaction by adding 2 µl of 0.25 M EDTA, pH 8.0.

   DNase treatment degrades the template. This step may not be necessary for probes to be used in hybridization analysis, but is worth doing to be on the safe side.

7. Measure the specific activity of the RNA by acid precipitation and remove unincorporated nucleotides by the spin-column procedure.

   The specific activity should be at least 7×10^8 dpm/µg, preferably $>10^9$ dpm/µg.

   The labeled probe can be stored at –20°C for 2 days before use.

Carry out hybridization analysis

8. Carry out the prehybridization incubation as described in steps 2 to 4 of the basic protocol, but use FPH solution and incubate at 42°C.

9. Replace the FPH solution with an equal volume of fresh prewarmed solution. Add the labeled probe and incubate overnight with rotation at 42°C.

   The probe concentration in the hybridization solution should be 1 to 5 ng/ml. Hybridizations in formamide solutions are carried out at lower temperatures than aqueous hybridizations. However, if background hybridization is a problem, raise the incubation temperature to 65°C.

10. Wash the membrane as described in steps 7 to 8 of the basic protocol.

11. Replace the wash solution with an equal volume of 2× SSC containing 25 µg/ml RNase A + 10 U/ml RNase T1; incubate with rotation for 30 min at room temperature.

    The RNase wash decreases background hybridization.

12. Carry out moderate- and high-stringency washes as desired, rinse the membrane in 2× SSC, and set up autoradiograph as in steps 9 to 12 of the basic protocol.

Table 2.10.1 Selection of Cloning Vectors Incorporating Promoters Bacteriophage RNA Polymerases

Vector	Size (bp)	Markers[a]	Promoters
pBluescript	2950	amp, *lacZ'*	T3, T7
pGEM series	2746-3223	amp, *lacZ'*	SP6, T7
pGEMEX-1	4200	amp	SP6, T3, T7
pSELECT-1	3422	tet, *lacZ'*	SP6, T7
pSP18, 19, 64, 65	2999-3010	amp	SP6
pSP70, 71, 72, 73	2417-2464	amp	SP6, T7
pSPORT1	4109	amp, *lacZ'*	SP6, T7
pT3/T7 series	2700, 2950	amp, *lacZ'*	T3, T7
pWE15	8800	amp, neo	T3, T7
pWE16	8800	amp, dhfr	T3, T7

[a]Abbreviations: amp, ampicillin resistance; dhfr, dihydrofolate reductase; *lacZ'*, β-galactosidase α-peptide; neo, neomycin phosphotransferase (kanamycin resistance); tet, tetracycline resistance.

REMOVAL OF PROBES FROM HYBRIDIZED MEMBRANES

SUPPORT PROTOCOL

If the DNA has been immobilized on the membrane by UV crosslinking (for uncharged nylon membranes) or by alkaline transfer (for positively charged nylon), the matrix-target DNA interaction (which is covalent in nature) is much stronger than the target-probe interaction (which results from hydrogen bonding). It is therefore possible to remove (or "strip off") the hybridized probe DNA without removing the membrane-bound target DNA. Nylon membranes can therefore be reused several times—a dozen reprobings are routinely possible. Hybridized probe DNA can also be stripped from nitrocellulose membranes, but the weakness of the hydrophobic interactions that bind the target DNA to the matrix, plus the fragility of nitrocellulose, limits the lifetime of these membranes to three reprobings at most.

This protocol describes three methods for probe stripping, in order of increasing harshness. The treatment that is needed depends on how tightly the probe anneals to the target, which in turn depends on the GC content of the probe and the number of base pairs that are formed. To start with, use the least harsh treatment, monitoring the results by autoradiography of the stripped membrane. If a hybridization signal can still be seen, move up to a harsher method. To strip probe from a northern membrane (with immobilized RNA), use the mild treatment only. Do not add NaOH to RNA; it will be destroyed.

Additional Materials

Mild stripping solution
Moderate stripping solution
0.4 M NaOH
0.1% (w/v) SDS, 100°C

CAUTION: Although the stripping solutions may not become highly radioactive, they should still be disposed of as radioactive waste.

1a. *Mild treatment:* Wash the membrane in several hundred milliliters of mild stripping solution for 2 hr at 65°C.

1b. *Moderate treatment:* Wash the membrane in 0.4 M NaOH for 30 min at 45°C. Then rinse twice in several hundred milliliters of moderate stripping solution for 10 min at room temperature.

1c. *Harsh t. ment:* Pour several hundred milliliters of boiling 0.1% SDS onto the membrane. Cool to room temperature.

If a membrane is to be reprobed, it must not be allowed to dry out between hybridization and stripping. If it becomes dry, the probe may bind to the matrix.

2. Place membrane on a sheet of dry Whatman 3MM filter paper and blot excess liquid with a second sheet. Wrap the membrane in plastic wrap and set up an autoradiograph.

If signal is still seen after autoradiography, rewash using harsher conditions.

3. The membrane can now be rehybridized. Alternatively, it can be dried and stored for later use.

Membranes can be stored dry between Whatman 3MM paper for several months at room temperature. For long-term storage, place the membranes in a desiccator at room temperature or 4°C.

REAGENTS AND SOLUTIONS

Aqueous prehybridization/hybridization (APH) solution

5× SSC (*APPENDIX 2*)
5× Denhardt solution (*APPENDIX 2*)
1% (w/v) SDS

Add 100 µg/ml denatured salmon sperm DNA (see below) just before use

Alternatives to Denhardt solution and denatured salmon sperm DNA as blocking agents are listed in Table 2.10.5 (see discussion in critical parameters).

Denatured salmon sperm DNA

Dissolve 10 mg Sigma type III salmon sperm DNA (sodium salt) in 1 ml water. Pass vigorously through a 17-G needle 20 times to shear the DNA. Place in a boiling water bath for 10 min, then chill. Use immediately or store at –20°C in small aliquots. If stored, reheat to 100°C for 5 min and chill on ice immediately before using.

Formamide prehybridization/hybridization (FPH) solution

5× SSC (*APPENDIX 2*)
5× Denhardt solution (*APPENDIX 2*)
50% (w/v) formamide
1% (w/v) SDS

Add 100 µg/ml denatured salmon sperm DNA (see above) just before use

Alternatives to Denhardt solution and denatured salmon sperm DNA as blocking agents are listed in Table 2.10.5 (see discussion in critical parameters).

Commercial formamide is usually satisfactory for use. If the liquid has a yellow color, deionize as follows: add 5 g of mixed-bed ion-exchange resin [e.g., Bio-Rad AG 501-X8 or 501-X8(D) resins] per 100 ml formamide, stir at room temperature for 1 hr, and filter through Whatman no. 1 paper.

CAUTION: *Formamide is a teratogen. Handle with care.*

Labeling buffer

200 mM Tris·Cl, pH 7.5
30 mM $MgCl_2$
10 mM spermidine

Mild stripping solution

5 mM Tris·Cl, pH 8.0
2 mM EDTA
0.1× Denhardt solution (*APPENDIX 2*)

Moderate stripping solution

200 mM Tris·Cl, pH 7.0
0.1× SSC (*APPENDIX 2*)
0.1% (w/v) SDS

Nucleotide mix

2.5 mM ATP
2.5 mM CTP
2.5 mM GTP
20 mM Tris·Cl, pH 7.5
Store at –20°C

COMMENTARY

Background Information

Hybridization between complementary polynucleotides was implicit in the Watson-Crick model for DNA structure and was initially exploited, via renaturation kinetics, as a means of studying genome complexity. In these early applications, the two hybridizing molecules were both in solution—an approach that is still utilized in "modern" techniques such nuclease protection transcript mapping (*UNITS 4.6 & 4.7*) and oligonucleotide-directed mutagenesis (Chapter 8). The innovative idea of immobilizing one hybridizing molecule on a solid support was first proposed by Denhardt (1966) and led to methods for identification of specific sequences in genomic DNA (dot blotting; *UNIT 2.9B*) and recombinant clones (*UNITS 6.3 & 6.4*). A second dimension was subsequently introduced by Southern (1975), who showed how DNA molecules contained in an electrophoresis gel could be transferred to a membrane (*UNIT 2.9A*), enabling genetic information relating to individual restriction fragments to be obtained by hybridization analysis.

Since the pioneering work of Denhardt and Southern, advances in membrane hybridization have been technical rather than conceptual. As reviewed by Dyson (1991), the detailed protocols have become more sophisticated, largely because of advances in understanding of the factors that influence hybrid stability and hybridization rate.

Hybrid stability is expressed as the melting temperature or T_m, which is the temperature at which the probe dissociates from the target DNA. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984):

$$T_m = 81.5°C + 16.6 (\log M) + 0.41(\%GC) - 0.61 (\%form) - 500/L$$

and for RNA-DNA hybrids from the equation of Casey and Davidson (1977):

$$T_m = 79.8°C + 18.5(\log M) + 0.58(\%GC) - 11.8(\%GC)^2 - 0.56(\%form) - 820/L$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The practical considerations that arise from these two equations are summarized Table 2.10.2A.

The second important consideration in hybridization analysis is the rate at which the hybrid is formed. Hybrid formation cannot occur until complementary regions of the two molecules become aligned, which occurs purely by chance; however, once a short nucleating region of the duplex has formed, the remaining sequences base-pair relatively rapidly. The rate at which the probe "finds" the target, which is influenced by a number of factors (Table 2.10.2B), is therefore the limiting step in hybrid formation (Britten and Davidson, 1985). However, in practical terms hybridization rate is less important than hybrid stability, as in most protocols hybridization is allowed to proceed for so long that factors influencing rate become immaterial.

Critical Parameters

To be successful, a hybridization experiment must meet two criteria:

(1) *Sensitivity.* Sufficient probe DNA must anneal to the target to produce a detectable signal after autoradiography.

(2) *Specificity.* After the last wash, the probe must be attached only to the desired target sequence (or, with heterologous probing, family of sequences).

Parameters influencing these two criteria are considered in turn, followed by other miscellaneous factors that affect hybridization.

Factors influencing sensitivity

The sensitivity of hybridization analysis is determined by how many labeled probe molecules attach to the target DNA. The greater the number of labeled probe molecules that anneal, the greater the intensity of the hybridization signal seen after autoradiography.

Probe specific activity. Of the various factors that influence sensitivity, the one that most frequently causes problems is the specific ac-

tivity of the probe. Modern labeling procedures, whether nick translation, random oligonucleotide priming (*UNIT 3.5*), or in vitro RNA synthesis (alternate protocol), routinely provide probes with a specific activity of $>10^8$ dpm/μg. This is the minimum specific activity that should be used in hybridization analysis of genomic DNA, even if the target sequences are multicopy. If the specific activity is $<10^8$ dpm/μg, hybridization signals will be weak or possibly undetectable, and no amount of adjusting the hybridization conditions will compensate. If there is a problem in obtaining a specific activity of $>10^8$ dpm/μg, it is important to troubleshoot the labeling protocol before attempting to use the probe in hybridization analysis.

If the probe is labeled to 10^8 to 10^9 dpm/μg, it will be able to detect as little as 0.5 pg of target DNA. Exactly what this means depends on the size of the genome being studied and the copy number of the target sequence. For human genomic DNA, 0.5 pg of a single-copy sequence 500 bp in length corresponds to 3.3 μg of total DNA. This is therefore the minimum amount of human DNA that should be used in a dot blot or Southern transfer if a single-copy gene is being sought.

Amount of target DNA. There is, however, a second argument that dictates that rather more than 3.3 μg of DNA should be loaded with each dot or Southern blot. During hybridization, genuine target sequences (100% homologous to the probe) and heterologous target sequences (related but not identical to the probe) compete with one another, with the homologous reactions always predominant. Ideally this competition should be maintained until the end of the incubation period so that maximum discrimination is seen between homologous and heterologous signals. This occurs only if the membrane-bound DNA is in excess, so that target sequences are continually competing for the available probe (Anderson and Young, 1985). If the probe is in excess then the homologous reaction may reach completion (i.e., all genuine target sites become filled) before the end of the incubation, leaving a period when only

Table 2.10.2 Factors Influencing Hybrid Stability and Hybridization Rate[a]

Factor	Influence
A. Hybrid stability[b]	
Ionic strength	T_m increases 16.6°C for each 10-fold increase in monovalent cations between 0.01 and 0.40 M NaCl
Base composition	AT base pairs are less stable than GC base pairs in aqueous solutions containing NaCl
Destabilizing agents	Each 1% of formamide reduces the T_m by about 0.6°C for a DNA-DNA hybrid. 6 M urea reduces the T_m by about 30°C
Mismatched base pairs	T_m is reduced by 1°C for each 1% of mismatching
Duplex length	Negligible effect with probes >500 bp
B. Hybridization rate[b]	
Temperature	Maximum rate occurs at 20-25°C below T_m for DNA-DNA hybrids, 10-15°C below T_m for DNA-RNA hybrids
Ionic strength	Optimal hybridization rate at 1.5 M Na^+
Destabilizing agents	50% formamide has no effect, but higher or lower concentrations reduce the hybridization rate
Mismatched base pairs	Each 10% of mismatching reduces the hybridization rate by a factor of two
Duplex length	Hybridization rate is directly proportional to duplex length
Viscosity	Increased viscosity increases the rate of membrane hybridization; 10% dextran sulfate increases rate by factor of ten
Probe complexity	Repetitive sequences increase the hybridization rate
Base composition	Little effect
pH	Little effect between pH 5.0 and pH 9.0

[a]This table is based on Brown (1991) with permission from BIOS Scientific Publishers.

[b]There have been relatively few studies of the factors influencing membrane hybridization. In several instances extrapolations are made from what is known about solution hybridization. This is probably reliable for hybrid stability, less so for hybridization rate.

heterologous hybridization occurring and during which discrimination between the homologous and heterologous signals becomes reduced. The problem is more significant with a double-stranded rather than a single-stranded probe, as with double-stranded probe reannealing between the two probe polynucleotides gradually reduces the effective probe concentration to such an extent that it always becomes limiting towards the end of the incubation.

In practical terms it is difficult to ensure that the membrane-bound DNA is in excess. The important factor is not just the absolute amount of DNA (which is dependent on the efficiency of immobilization and how many times the membrane has been reprobed) but also the proportion of the DNA that is composed of sequences (homologous and heterologous) able to hybridize to the probe. Rather than attempting complex calculations whose results may have factor-of-ten errors, it is advisable simply to blot as much DNA as possible: 10 µg is sufficient with most genomes. Assuming that the probe is labeled adequately and used at the correct concentration in the hybridization solution, a clear result will be obtained after autoradiography for a few hours with a simple genome (e.g., yeast DNA) or a few days with a more complex one (e.g., human DNA).

Labels other than ^{32}P. The discussion so far has assumed that the probe is labeled with ^{32}P. The lower emission energy of ^{35}S results in reduced sensitivity, and this isotope is in general unsuitable for hybridization analysis of genomic DNA. ^{35}S can be used only if the blotted DNA is exceptionally noncomplex (e.g., restricted plasmid DNA), or if the DNA is highly concentrated (e.g., colony and plaque blots; *UNIT 6.3*). Note that a membrane hybridized to a ^{35}S-labeled probe has to be dried before autoradiography, so probe stripping is not possible.

Nonradioactive probes are a more realistic option for hybridization analysis of genomic DNA and are becoming increasingly popular, as the problems involved in their use are gradually ironed out. Their advantages include greater safety, the fact that large amounts of probe can be prepared in one batch and stored for years, and the rapidity of the detection protocols. Their main disadvantage is that the sensitivity of most nonradioactive detection systems is lower than that of ^{32}P autoradiography, which means that the blot and hybridization have to be carried out at maximum efficiency if a satisfactory signal is to be seen. For details on hybridization analysis with nonradioactive probes, see *UNITS 3.1b & 3.19* and Mundy et al. (1991).

Using an inert polymer to increase sensitivity. In addition to adjusting the parameters discussed above, an improvement in sensitivity can also be achieved by adding an inert polymer such as 10% (w/v) dextran sulfate (molecular weight 500,000) or 8% (w/v) PEG 6000 to the hybridization solution. Both induce an increase in hybridization signal, 10-fold with a single-stranded probe and as much as 100-fold if the probe is double-stranded (Wahl et al., 1979; Amasino, 1986). The improvement is thought to arise from formation of interlocked meshes of probe molecules, which anneal en masse at target sites. Increased hybridization signals are a major bonus in detecting single-copy sequences in complex genomes, but this must be balanced with the fact that the polymers make the hybridization solutions very viscous and difficult to handle.

Factors influencing specificity

Ensuring specificity in homologous hybridization experiments. The hybridization incubation is carried out in a high-salt solution that promotes base-pairing between probe and target sequences. In 5× SSC, the T_m for genomic DNA with a GC content of 50% is about 96°C. Hybridization is normally carried out at 68°C, so the specificity of the experiment is not determined at this stage. Specificity is the function of post-hybridization washes, the critical parameters being the ionic strength of the final wash solution and the temperature at which this wash is carried out.

The highly stringent wash conditions described in the basic and alternate protocols should destabilize all mismatched heteroduplexes, so that hybridization signals are obtained only from sequences that are perfectly homologous to the probe. For DNA and RNA probes (as opposed to oligonucleotides), problems with lack of specificity after the highly stringent wash occur only if the hybridizing sequences are very GC-rich, resulting in a relatively high T_m. If the high-stringency wash does not remove all nonspecific hybridization, temperature can be increased by a few degrees. The equations above for calculating T_m can be used as a guide for selecting the correct temperature for the final wash, but trial and error is more reliable. Note that a membrane that has been autoradiographed can be rewashed at a higher stringency and put back to expose again, the only limitation being decay of the label and the need for a longer exposure the second time.

Table 2.10.3 High-Salt Solutions Used in Hybridization Analysis

Stock solution	Composition
20× SSC	3.0 M NaCl/0.3 M trisodium citrate
20× SSPE[a]	3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7
Phosphate solution[b]	1 M $NaHPO_4$, pH 7.2[c]

[a]SSC may be replaced with the same concentration of SSPE in all protocols.

[b]Prehybridize and hybridize with 0.5 M $NaHPO_4$ (pH 7.2)/1 mM EDTA/7% SDS [or 50% formamide/0.25 M $NaHPO_4$ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS]; perform moderate-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/5% SDS; perform high-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/1% SDS.

[c]Dissolve 134 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter water, then add 4 ml 85% H_3PO_4. The resulting solution is 1 M Na^+, pH 7.2.

Designing washes for heterologous hybridization. Calculations of T_m become more critical if heterologous probing is being attempted. If the aim is to identify sequences that are merely related, not identical, to the probe (e.g., members of a multigene family, or a similar gene in a second organism), then it is useful to have an idea of the degree of mismatching that will be tolerated by a "moderate-" or "low-" stringency wash. The best way to approach this is to first establish the lowest temperature at which only homologous hybridization occurs with a particular SSC concentration. Then assume that 1% mismatching results in a 1°C decrease in the T_m (Bonner et al., 1973) and reduce the temperature of the final wash accordingly (for example, if sequences with ≥90% similarity with the probe are being sought, decrease the final wash temperature by 10°C). If the desired degree of mismatching results in a wash temperature of <45°C, then it is best to increase the SSC concentration so that a higher temperature can be used. Doubling the SSC concentration results in a ~17°C increase in T_m, so washes at 45°C in 0.1× SSC and 62°C in 0.2× SSC are roughly equivalent. Note that in these extreme cases it may also be necessary to reduce the hybridization temperature to as low as 45°C (aqueous solution) or 32°C (formamide solution).

This approach sometimes works extremely well (as shown when the heterologous targets are eventually sequenced), but the assumption that a 1% degree of mismatching reduces the T_m of a heteroduplex by 1°C is very approximate. Base composition and mismatch distribution influence the actual change in T_m, which can be anything between 0.5° and 1.5°C per 1% mismatch (Hyman et al., 1973). Unfortunately trial and error is the only alternative to the "rational" approach described here.

Other parameters relevant to hybridization analysis

Length of prehybridization and hybridization incubations. The protocols recommend prehybridization for 3 hr with nitrocellulose and 15 min for nylon membranes. Inadequate prehybridization can lead to high backgrounds, so these times should not be reduced. They can, however, be extended without problem.

Hybridizations are usually carried out overnight. This is a rather sloppy aspect of the procedure, because time can have an important influence on the result, especially if, as described above, an excess amount of a single-stranded probe is being used. The difficulties in assigning values to the parameters needed to calculate optimum hybridization time has led to the standard "overnight" incubation, which in fact is suitable for most purposes. The exception is when hybridization is being taken to its limits, for instance in detection of single-copy sequences in human DNA, when longer hybridization times (up to 24 hr) may improve sensitivity if a single-stranded probe is being used. Note that this does not apply to double-stranded probes, as gradual reannealing results in only minimal amounts of a double-stranded probe being free to hybridize after ~8 hr of incubation.

Formamide hybridization buffers. Formamide destabilizes nucleic acid duplexes, reducing the T_m by an average of 0.6°C per 1% formamide for a DNA-DNA hybrid (Meinkoth and Wahl, 1984) and rather less for a DNA-RNA hybrid (Casey and Davidson, 1977; Kafatos et al., 1979). It can be used at 50% concentration in the hybridization solution, reducing the T_m so that the incubation can be carried out at a lower temperature than needed with an aqueous solution. Originally formamide was used with nitrocellulose membranes as a means of prolonging their lifetime, as the

lower hybridization tempera ,results in less removal of target DNA from the matrix. More recently formamide has found a second use in reduction of heterologous background hybridization with RNA probes. RNA-DNA hybrids are relatively strong, and heterologous duplexes remain stable even at high temperatures. The destabilizing effect of formamide is therefore utilized to maximize the discrimination between homologous and heterologous hybridization with RNA probes.

Formamide probably confers no major advantage on DNA-DNA hybridization with a nylon membrane. In fact it introduces two problems, the hazardous nature of the chemical itself, and an apparent reduction in hybridization rate. The latter point is controversial (Hutton, 1977), but for equivalent sensitivity a formamide hybridization reaction usually has to incubate for longer than an aqueous one.

Alternatives to SSC. Although SSC has been used in hybridization solutions for many years, there is nothing sacrosanct about the formulation, and other salt solutions can be employed (Table 2.10.3). There is little to choose between these alternatives. SSPE and phosphate solutions have a greater buffering power and may confer an advantage in formamide hybridization solutions. Alternatively, the buffering power of SSC can be increased by adding 0.3% (w/v) tetrasodium pyrophosphate.

Probe length. Probe length has a major influence on the rate of duplex formation in solution hybridization (Wetmur and Davidson, 1986), but the effect is less marked when the target DNA is immobilized. In membrane hybridization a more important factor is the specificity of the probe. The probe should never be too long (>1000 bp), as this increases the chance of heterologous duplexes remaining stable during a high-stringency wash. Neither should the probe contain extensive vector sequences, as these can hybridize to their own target sites, wrecking the specificity of the experiment.

Mechanics of hybridization. Traditionally hybridization has been carried out in plastic bags. This technique is messy, radiochemical spills being almost unavoidable, and can lead to detrimental contact effects if too many membranes are hybridized in a single bag. Hybridization incubators are now available from a number of companies and are recommended as a distinct advance over the plastic bag technology. Rotation of the hybridization tube results in excellent mixing, reducing hot spots caused by bubbles and dust and leading to very evenly hybridized membranes. Gc quality results are possible even when ten or more minigel Southerns are hybridized in a single 8.5 × 3.0–inch tube.

If bags are used, they should be of stiff plastic to prevent the sides collapsing on to the membrane, which will lead to high background. The volume of hybridization solution should be sufficient to fill the bag, and no more than two membranes should be hybridized in each bag.

Troubleshooting

Problems in blotting and hybridization reveal themselves when the autoradiograph is developed. A guide to the commonest problems and how to solve them is given in Table 2.10.4 (based on Dyson, 1991).

A particularly troublesome problem is high background signal across the entire membrane. This is due to the probe attaching to nucleic acid binding sites on the membrane surface, the same sites that bind DNA during the blotting procedure. Prehybridization/hybridization solutions contain reagents that block these sites and hence reduce background hybridization. The most popular blocking agent is Denhardt solution, which contains three polymeric compounds (Ficoll, polyvinylpyrrolidone, and BSA) that compete with nucleic acids for the membrane-binding sites. The formulations used in the basic and alternate protocols also include denatured salmon sperm DNA (any complex DNA that is nonhomologous with the target is acceptable) which also competes with the probe for the membrane sites. Blocking agents are included in the prehybridization solution to give them a head start over the probe. With a nylon membrane, the blocking agents may have to be left out of the hybridization solution, as they can interfere with the probe-target interaction. When the membranes are washed, the Denhardt solution and salmon sperm DNA are replaced with SDS, which acts as a blocking agent at concentrations ≥1%.

Other blocking agents can also be used (Table 2.10.5). With DNA blots, the main alternatives to Denhardt are heparin (Singh and Jones, 1984) and milk powder (BLOTTO; Johnson et al., 1984), although Denhardt is generally more effective, at least with nylon membranes. Note that BLOTTO contains RNases and so can be used only in DNA-DNA hybridizations. With an RNA probe, denatured salmon sperm DNA is sometimes replaced by 100 µg/ml yeast tRNA, which has the advantage that it does not need to be sheared before

Table 2.10.4 Troubleshooting Guide for DNA Blotting and Hybridization Analysis[a]

Problem	Possible cause[b]	Solution
Poor signal	Probe specific activity too low	Check labeling protocol if specific activity is $<10^8$ dpm/μg.
	Inadequate depurination	Check depurination if transfer of DNA >5 kb is poor.
	Inadequate transfer buffer	1. Check that 20× SSC has been used as the transfer solution if small DNA fragments are retained inefficiently when transferring to nitrocellulose. 2. With some brands of nylon membrane, add 2 mM Sarkosyl to the transfer buffer. 3. Try alkaline blotting to a positively charged nylon membrane.
	Not enough target DNA	Refer to text for recommendations regarding amount of target DNA to load per blot.
	Poor immobilization of DNA	See recommendations in *UNIT 2.9A* commentary.
	Transfer time too short	See recommendations in *UNIT 2.9A* commentary.
	Inefficient transfer system	Consider vacuum blotting as an alternative to capillary transfer.
	Probe concentration too low	1. Check that the correct amount of DNA has been used in the labeling reaction. 2. Check recovery of the probe after removal of unincorporated nucleotides. 3. Use 10% dextran sulfate in the hybridization solution. 4. Change to a single-stranded probe, as reannealing of a double-stranded probe reduces its effective concentration to zero after hybridization for 8 hr.
	Incomplete denaturation of probe	Denature as described in the protocols.
	Incomplete denaturation of target DNA	When dot or slot blotting, use the double denaturation methods described in *UNIT 2.9B*, or blot onto positively charged nylon.
	Blocking agents interfering with the target-probe interaction	If using a nylon membrane, leave the blocking agents out of the hybridization solution.
	Final wash was too stringent	Use a lower temperature or higher salt concentration. If necessary, estimate T_m as described in *UNIT 6.4*.
	Hybridization temperature too low with an RNA probe	Increase hybridization temperature to 65°C in the presence of formamide (see alternate protocol).
	Hybridization time too short	If using formamide with a DNA probe, increase the hybridization time to 24 hr.
	Inappropriate membrane	Check the target molecules are not too short to be retained efficiently by the membrane type (see Table 2.9.1).

continued

Table 2.10.4 Troubleshooting Guide for DNA Blotting and Hybridization Analysis[a], continued

Problem	Possible cause[b]	Solution
	Problems with electroblotting	Make sure no bubbles are trapped in the filter-paper stack. Soak the filter papers thoroughly in TBE before assembling the blot. Used uncharged rather than charged nylon.
Spotty background	Unincorporated nucleotides not removed from labeled probe	Follow protocols described in *UNIT 3.4*.
	Particles in the hybridization buffer	Filter the relevant solution(s).
	Agarose dried on the membrane	Rinse membrane in 2× SSC after blotting.
	Baking or UV cross-linking when membrane contains high salt	Rinse membrane in 2× SSC after blotting.
Patchy or generally high background	Insufficient blocking agents	See text for of discussion of extra/alternative blocking agents.
	Part of the membrane was allowed to dry out during hybridization or washing	Avoid by increasing the volume of solutions if necessary.
	Membranes adhered during hybridization or washing	Do not hybridize too many membranes at once (ten minigel blots for a hybridization tube, two for a bag is maximum).
	Bubbles in a hybridization bag	If using a bag, fill completely so there are no bubbles.
	Walls of hybridization bag collapsed on to membrane	Use a stiff plastic bag; increase volume of hybridization solution.
	Not enough wash solution	Increase volume of wash solution to 2 ml/10 cm^2 of membrane.
	Hybridization temperature too low with an RNA probe	Increase hybridization temperature to 65°C in the presence of formamide (see alternate protocol).
	Formamide needs to be deionized	Although commercial formamide is usually satisfactory, background may be reduced by deionizing immediately before use.
	Labeled probe molecules are too short	1. Use a ^{32}P-labeled probe as soon as possible after labeling, as radiolysis can result in fragmentation. 2. Reduce amount of DNase I used in nick translation (*UNIT 3.5*).
	Probe concentration too high	Check that the correct amount of DNA has been used in the labeling reaction.
	Inadequate prehybridization	Prehybridize for at least 3 hr with nitrocellulose or 15 min for nylon.
	Probe not denatured	Denature as described in the protocols.
	Inappropriate membrane type	If using a nonradiocative label, check that the membrane is compatible with the detection system.
	Hybridization with dextran sulfate	Dextran sulfate sometimes causes background hybridization. Place the membrane between Schleicher and Schuell no. 589 WH paper during hybridization, and increase volume of hybridization solution (including dextran sulfate) by 2.5%.

continued

Table 2.10.4 Troubleshooting Guide for DNA Blotting and Hybridization Analysis[a], continued

Problem	Possible cause[b]	Solution
	Not enough SDS in wash solutions	Check the solutions are made up correctly.
Extra bands	Final wash was not stringent enough	Use a higher temperature or lower salt concentration. If necessary, estimate T_m as described in *UNIT 6.4*.
	Probe contains nonspecific sequences (e.g., vector DNA)	Purify shortest fragment that contains the desired sequence.
	Target DNA is not completely restriction digested	Check the restriction digestion (*UNIT 3.1*).
	Formamide not used with an RNA probe	RNA-DNA hybrids are relatively strong but are destabilized if formamide is used in the hybridization solution.
Nonspecific background in one or more tracks	Probe is contaminated with genomic DNA	Check purification of probe DNA. The problem is more severe when probes are labeled by random printing. Change to nick translation.
	Insufficient blocking agents	See text for of discussion of extra/alternative blocking agents.
	Final wash did not approach the desired stringency	Use a higher temperature or lower salt concentration. If necessary, estimate T_m as described in *UNIT 6.4*.
	Probe too short	Sometimes a problem with probes labeled by random priming. Change to nick translation.
Cannot remove probe after hybridization	Membrane dried out after hybridization	Make sure the membrane is stored moist between hybridization and stripping.
Decrease in signal intensity when reprobed	Poor retention of target DNA during probe stripping	1. Check calibration of UV source if cross-linking on nylon. 2. Use a less harsh stripping method (support protocol).

[a]Based on Dyson (1991).

[b]Within each category, possible causes are listed in decreasing order of likelihood.

use. If a cDNA clone is used as the probe, or for the in vitro synthesis of an RNA probe, then blockage of sites with high affinity for poly(A)$^+$ sequences often reduces background. This is achieved by using 10 µg/ml of poly(A) DNA as the blocking agent.

Anticipated Results

Using either a nitrocellulose or nylon membrane and a probe labeled to $\geq 10^8$ dpm/µg, there should be no difficulty in detecting 10 pg of a single copy sequence in human DNA after 24 hr autoradiography.

Time Considerations

The hybridization experiment can be completed in 24 hr, the bulk of this being taken up by the overnight incubation. Prehybridization takes 3 hr for a nitrocellulose membrane or 15 min for nylon. Post-hybridization washing to high stringency can usually be completed in 1.5 hr. If a single-copy sequence in human DNA is being probed, the hybridization step may be extended to 24 hr, with a concomitant increase in the length of the experiment as a whole.

The length of time needed for autoradiography depends on the abundance of the target sequences in the blotted DNA. Adequate exposure can take anything from overnight to several days.

Table 2.10.5 Alternatives to Denhardt/Denatured Salmon Sperm DNA as Blocking Agents in DNA Hybridization[a]

Blocking agent	Composition	Storage and use
BLOTTO	5% (w/v) nonfat dried milk/0.02% (w/v) NaN_3 in H_2O	Store at 4°C; use at 4% final concentration
Heparin (porcine grade II)	50 mg/ml in 4× SSC	Store at 4°C. Use at 500 µg/ml with dextran sulfate or 50 µg/ml without
Yeast tRNA	10 mg/ml in H_2O	Store at 4°C; use at 100 µg/ml
Homopolymer DNA	1 mg/ml poly(A) or poly(C) in H_2O	Store at 4°C; use at 10 µg/ml in water; appropriate targets: poly(A) for AT-rich DNA, poly(C) for GC-rich DNA

[a]This table is based on Brown (1991) with permission from BIOS Scientific Publishers.

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Provides a detailed account of factors influencing hybridization.

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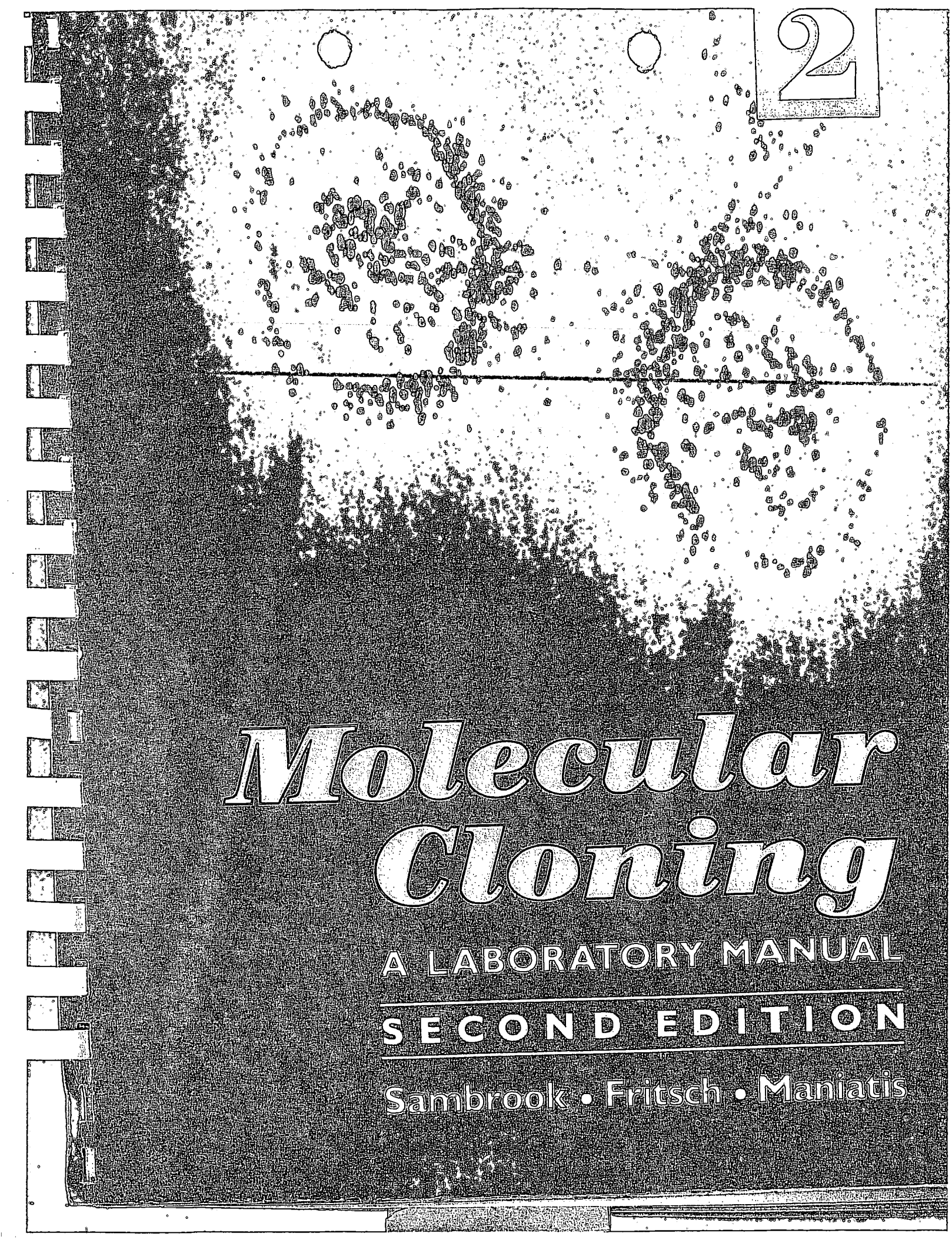
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A LABORATORY MANUAL
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Conditions for Hybridization of Oligonucleotide Probes

When using oligonucleotides as probes, the aim is to find conditions that are stringent enough to guarantee specificity and sufficiently flexible to allow formation of stable hybrids at an acceptable rate. For DNA molecules more than 200 nucleotides in length, hybridization is usually carried out at 15–25°C below the calculated melting temperature (T_m) of a perfect hybrid. However, as the length of the probe is decreased, the T_m is lowered to the point where it is often impractical to carry out hybridization at $T_m - 25$°C. Typically, therefore, hybridization with synthetic oligonucleotides is carried out under conditions that are only 5–10°C below the T_m. Although such stringent conditions reduce the number of mismatched clones that are detected with short oligonucleotide probes, they have the less desirable consequence of reducing the rate at which perfect hybrids form.

Hybrids formed between DNA molecules more than 200 nucleotides in length are completely stable for all practical purposes. The chances that such a long stretch of double helix will unwind at temperatures 15–25°C below the T_m are extremely small. However, hybrids (even perfect hybrids) formed between short oligonucleotides and their target sequences at 5–10°C below the T_m are far easier to unwind, and hybridization reactions of this type can be regarded as reversible. This has important practical consequences. Whereas hybrids formed between longer DNA molecules are essentially stable under the conditions used for posthybridization washing, hybrids (even perfect hybrids) involving short oligonucleotides are not. Posthybridization washing of such hybrids must therefore be carried out rapidly so that the probe does not dissociate from its target sequence. For this reason, hybridizations with short oligonucleotides should be carried out under stringent conditions (5–10°C below the T_m) using high concentrations (0.1–1.0 pmole/ml) of probe. When only one or a small number of oligonucleotides (<8) are used as probes, the annealing reaction rapidly reaches equilibrium, and hybridization should therefore be terminated after 3 or 4 hours. More complex mixtures, in which the concentration of each oligonucleotide is comparatively low, require hybridization to be carried out for proportionately longer periods. For example, mixtures of 32 or more oligonucleotides should be hybridized for 1–2 days. Posthybridization washing should be carried out for brief periods of time, initially under conditions of low stringency and then under conditions of stringency equal to those used for hybridization.

CALCULATING MELTING TEMPERATURES FOR PERFECTLY MATCHED HYBRIDS BETWEEN OLIGONUCLEOTIDES AND THEIR TARGET SEQUENCES

When using single oligonucleotides that match the target sequence perfectly, hybridization conditions can easily be derived from the calculated T_m of the hybrid. For oligonucleotides shorter than 18 nucleotides, the T_m of the hybrid can be estimated by multiplying the number of A + T residues in the hybrid by 2°C and the number of G + C residues by 4°C and adding the two numbers (Itakura et al. 1984). However, this method overestimates the T_m of hybrids involving longer oligonucleotides.

A different approach has been taken by E. Fritsch (unpubl.), who found that the equation originally used to calculate the relationship between G + C content, ionic strength of the hybridization solution, and the T_m of long DNA molecules (Bolton and McCarthy 1962):

$$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\,G + C) - (600/N),$$

where N = chain length, predicts reasonably well the T_m for oligonucleotides as long as 60–70 nucleotides and as short as 14 nucleotides.

ESTIMATING THE EFFECTS OF MISMATCHES

Perhaps surprisingly, the classic formula (Bonner et al. 1973) to calculate the effect of mismatches on the stability of long DNA hybrids holds reasonably well for hybrids involving short oligonucleotides: For every 1% of mismatching of bases in a double-stranded DNA, there is a reduction of T_m by 1–1.5°C. However, the precise effect of mismatches depends on the G + C content of the oligonucleotide and, even more critically, on the distribution of mismatched bases in the double-stranded DNA. Mismatches in the middle of the oligonucleotide are far more deleterious than mismatches at the ends. Therefore, the method of estimation given above should only be used as a rough guide until a systematic study of all types of mismatches in a variety of contexts leads to more precise methods of estimation. If appropriate target DNA has been cloned, the effect of mismatches on T_m can be determined empirically (see pages 11.55–11.57).

HYBRIDIZATION OF POOLS OF OLIGONUCLEOTIDES

It is easy to calculate accurately the T_m of a perfectly matched hybrid formed between a single oligonucleotide and its target sequence. However, when using pools of oligonucleotides whose members have greatly different contents of G + C, it is impossible to estimate a consensus T_m. Because it is not possible to know which member of the pool will match the target sequence perfectly, conditions must be used that allow the oligonucleotide with the lowest content of G + C to hybridize efficiently. Usually, conditions are chosen to be 2°C below the calculated T_m of the most A/T-rich member of the pool (Suggs et al. 1981b). However, the use of such "lowest common denominator" conditions can lead to a number of false positives, because mismatched hybrids formed by oligonucleotides of higher G + C content may be more stable than a perfectly matched hybrid formed by the correct oligonucleotide. In most cases, this problem is not serious, since the number of positive clones obtained by screening cDNA libraries with pools of oligonucleotides is usually quite manageable. It is therefore possible to easily distinguish false positives from true positives by another test (e.g., DNA sequencing or hybridization with a second pool of oligonucleotides corresponding to another segment of amino acid sequence).

In those cases when the number of positives is unacceptably high, it may be worthwhile to consider using hybridization solvents that contain the quaternary alkylammonium salts tetraethylammonium chloride (TEACl) or tetramethylammonium chloride (TMACl) instead of sodium chloride (Melchior and von Hippel 1973; Jacobs et al. 1985, 1988; Wood et al. 1985; Gitschier et al. 1986; Wozney 1989). In these solvents, the T_m of a hybrid is independent of its base composition and dependent primarily on its length. Thus, by choosing a temperature for hybridization appropriate for the lengths of the oligonucleotides in a pool, the effects of potential mismatches can be minimized.

It is important to obtain an accurate estimate of the T_m in TMACl or TEACl before using pools of oligonucleotides to screen cDNA or genomic DNA libraries. Jacobs et al. (1988) measured the T_i (the irreversible melting temperature of the hybrid formed between the probe and its target sequence) as a function of chain length for a number of oligonucleotides of differing G + C content in solvents containing either sodium or tetramethylammonium ions. Hybrids involving oligonucleotides 16 and 19 nucleotides in length melt over a smaller range of temperature in solvents containing TMACl than in solvents containing sodium salts (3°C for TMACl vs. 17°C for SSC when hybridizing 16-mers; 5°C for TMACl vs. 20°C for SSC when hybridizing 19-mers). For 14-mers, the effect is much less dramatic (7°C for TMACl vs. 9°C for SSC). Similar, but less extensive, data are available for solvents containing TEACl (Jacobs et al. 1988).

The optimal temperature for hybridization is usually chosen to be 5°C below the T_i for the given chain length. The recommended hybridization temperature for 17-mers in 3 M TMACl is 48–50°C; for 19-mers, it is 55–57°C; and for 20-mers, it is 58–66°C. Three points are worth emphasizing. First, the T_is of hybrids are uniformly 15–20°C higher in solvents containing TMACl than in solvents containing TEACl. The higher T_i in solvents containing TMACl allows hybridization to be performed at temperatures that

suppress nonspecific adsorption of the probe to solid supports (such as nylon membranes), resulting in lower nonspecific backgrounds. Second, hybridization solvents containing TMACl do not have significant advantages over those containing sodium ions until the length of the oligonucleotide exceeds 16 nucleotides. Finally, the data have been extensively examined for 16-mers, 19-mers, and, in previous studies, for much longer DNA molecules (Melchior and von Hippel 1973). It is currently an untested assumption that the same beneficial effect will be seen for DNA molecules of all intermediate lengths.

Preparation and Use of Solvents Containing Quaternary Alkylammonium Salts

1. Prepare a 6 M solution of tetramethylammonium chloride (TMACl) or a 3 M solution of tetraethylammonium chloride (TEACl) in H_2O. (TMACl and TEACl are available from Aldrich.)

2. Add activated charcoal to a final concentration of approximately 10% and stir for 20–30 minutes.

3. Allow the charcoal to settle, and then filter the solution of TMACl or TEACl through a Whatman No. 1 paper.

4. Filter the solution through a nitrocellulose filter (e.g., Nalge, 0.45-micron pore size). Store the filtered solution in dark bottles at room temperature.

5. Measure the refractive index of the solution, and calculate the precise concentration of the solution from the equation:

   $$C = (n - 1.331)/0.018$$

   where C = molar concentration of quaternary alkylammonium salt and n = refractive index.

6. Prehybridize nitrocellulose filters or nylon membranes for 2–6 hours in oligonucleotide prehybridization solution.

Oligonucleotide prehybridization solution

6× SSC (or 6× SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

7. Prepare the quaternary alkylammonium solution to be used for hybridization:

Quaternary alkylammonium hybridization solution

3.0 M TMACl or 2.4 M TEACl
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 7.6)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
0.1% nonfat dried milk

Notes

i. Nitrocellulose filters are not stable when hybridization is carried out for extended periods of time in solvents containing TMACl or TEACl. Nylon membranes are much better suited for this purpose.

ii. Posthybridization washing is usually carried out initially with solutions containing sodium salts (e.g., 6 × SSC) rather than quaternary alkylammonium salts. If additional stringent washes are required, rinse the filters first with quaternary alkylammonium hybridization solution (without DNA or nonfat dried milk) at room temperature and then briefly (5–10 minutes) with the same solution at $T_i - 5°C$.

HYBRIDIZATION OF GUESSMERS

Perhaps the most critical step in the use of guessmers is the choice of conditions for hybridization. The temperature should be high enough to suppress hybridization of the probe to incorrect sequences but must not be so high as to prevent hybridization to the correct sequence, even though it may be mismatched. Before using an oligonucleotide to screen a library, it is therefore advisable to perform a series of trial experiments in which a series of northern or genomic Southern hybridizations are carried out under different degrees of stringency (Anderson and Kingston 1983; Wood et al. 1984). A set of theoretical curves relating the temperature of the washing solution to the length and homology of the probe is given in Lathe (1985). Using these curves as a guide, determine the optimal conditions for detection of sequences complementary to the probe by hybridizing the oligonucleotide to a series of nitrocellulose filters or nylon membranes at different temperatures. The filters are washed extensively in 6× SSC at room temperature and then briefly (5–10 minutes in 6× SSC) at the temperature used for hybridization. This method, in which both hybridization and washing are carried out under the same conditions of temperature and ionic strength, appears to be more discriminating than the more commonly used procedure of hybridizing under conditions of lower stringency and washing under conditions of higher stringency.

If trial experiments are not possible, attempt to estimate the melting temperature (T_m) as follows:

1. Calculate the minimum G + C content of the oligonucleotide assuming that A or T is present at all positions of ambiguity.
2. Using the following formula, calculate the T_m of a double-stranded DNA with the calculated G + C content:

   $$T_m = 81.5 - 16.6(\log_{10}[Na^+]) + 0.41(\%\ G + C) - (600/N)$$

   where N = chain length.
3. Calculate the maximum amount of possible mismatch assuming that all choices of degenerate codons are incorrect. Subtract 1°C from the calculated T_m for each 1% of mismatch. The resulting number should be the T_m of a maximally mismatched hybrid formed between the probe and its target DNA sequence.

In the absence of information from trial experiments, hybridization and washing should be carried out at 5–10°C below the estimated T_m. Almost certainly, the actual T_m will be higher than that predicted by this worst-case calculation. If the bases used at positions of ambiguity were chosen at random, one out of four should be correct, and approximately half of these would be expected to be G or C. The observed T_m should therefore be significantly higher than that estimated. However, to minimize the risk of missing the clone of interest, it is best to hybridize and wash at several degrees below the T_m estimated as described above. If, under these conditions, the probe hybridizes indiscriminately, repeat the hybridization at a higher temperature or wash under conditions of higher stringency.

Before proceeding to screen an entire cDNA or genomic DNA library, it is advisable to carry out a series of pilot experiments in which the probe is hybridized under different conditions to small aliquots (perhaps 5000–10,000 clones) of the library that is to be screened. The results of these experiments should allow you to choose conditions for large-scale screening that are just stringent enough to eliminate nondiscriminate hybridization of the probe to the vast majority of clones in the library.

Hybridization of guessmers in solvents that contain quaternary alkylammonium salts has not been investigated.

HYBRIDIZATION OF OLIGONUCLEOTIDES THAT CONTAIN A NEUTRAL BASE AT POSITIONS OF DEGENERACY

Although the conditions for hybridization of probes that contain the neutral base inosine have not been extensively explored, it is possible to make a conservative estimate of the melting temperature (T_m) as follows:

1. Subtract the number of inosine residues from the total number of nucleotides in the probe to give a value S.
2. Calculate the G + C content of S.
3. Estimate the T_m of a perfect hybrid involving S using the equation on page 11.52.
4. Use conditions for hybridization that are 15–20°C below the estimated T_m.

The T_m of hybrids involving oligonucleotides that contain neutral bases can also be estimated empirically as described on pages 11.55–11.57. Hybridization of such oligonucleotides in solvents containing quaternary alkylammonium salts has not been investigated.

EMPIRICAL DETERMINATION OF MELTING TEMPERATURE

The melting temperature (T_m) of an oligonucleotide hybridized to a target sequence can be determined by the procedure described below. The protocol actually measures the temperature at which dissociation of the double-stranded DNA becomes irreversible (T_i) in nonequilibrium conditions that do not favor rehybridization of the released probe to the target. The optimal temperature for hybridization is then determined on the basis of this value. The procedure requires a cloned target sequence that is complementary (perfectly or imperfectly, depending on the experiment) to the oligonucleotide probe. In most cases, a target sequence is not available from "natural" sources and must be synthesized chemically. The best synthetic target sequences consist of two oligonucleotides that are partially complementary. After annealing, these oligonucleotides form a double-stranded region that contains the target sequence. The sequences of the protruding ends are designed to allow the target DNA to be cloned easily in bacteriophage M13 vectors. Single-stranded DNA of the appropriate orientation prepared from the resulting clones (see Chapter 4) can be used in hybridization experiments as described below. It can also be used as a template for dideoxy-mediated chain-termination sequencing (see Chapter 13) if it is necessary to check that the sequence of the target DNA is correct.

1. Label 1–10 pmoles of the oligonucleotide to be used as a probe by phosphorylation (see pages 11.31–11.32), and remove excess unincorporated [γ-^{32}P]ATP by one of the methods described on pages 11.33–11.39.

2. Using a paper punch, prepare four small circles (diameter 3–4 mm) of a solid support (nitrocellulose filter or nylon membrane) used for hybridization. Arrange the small circles on a piece of Parafilm. Mark two of the filters with a soft-lead pencil.

3. Apply approximately 100 ng of target single-stranded DNA in a volume of 1–3 μl of 2× SSC to each of the marked filters. Apply an equal amount of vector DNA to the unmarked filters. After the fluid has dried, use blunt-ended forceps (e.g., Millipore forceps) to remove the two sets of filters from the Parafilm, and place them between sheets of Whatman 3MM paper. Fix the DNAs to the filters by baking for 1–2 hours at 80°C in a vacuum oven.

   If the target DNA has been cloned into a plasmid, linearize the vector by digestion with a restriction enzyme that does not cleave within the target sequences. Purify the resulting double-stranded DNA by extraction with phenol:chloroform and precipitation with ethanol. Dissolve the DNA in 2× SSC at a concentration of 500 ng/μl. Apply the solution of DNA to the filters prepared as described above, and then, using blunt-ended forceps, transfer the filters to a sheet of 3MM paper saturated with denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 5–10 minutes. Move the filters to a fresh sheet of 3MM paper saturated with neutralizing solution (0.5 M Tris · Cl [pH 7.4], 1.5 M NaCl) for 10 minutes. Transfer the filters to a dry sheet of 3MM paper, and leave them at room temperature until all of the fluid has evaporated. Bake the filters as described above.

   Overbaking can cause the filters to become brittle. In addition, filters that have not been completely neutralized turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

4. Using blunt-ended forceps, transfer all of the filters to a polyethylene tube that contains 2 ml of oligonucleotide prehybridization solution. Seal the tube and incubate, with occasional shaking, at a temperature estimated to be $T_m - 25°C$ for the solvent being used (see Note i). After 2 hours, add radiolabeled oligonucleotide to the prehybridization solution. The final concentration of oligonucleotide should be approximately 1 pmole/ml. Continue incubation at $T_m - 25°C$ for a further 2–4 hours, with occasional shaking.

Oligonucleotide prehybridization solution

6 × SSC (or 6 × SSPE)
0.01 M sodium phosphate (pH 6.8)
1 mM EDTA (pH 8.0)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA (see Appendix B)
0.1% nonfat dried milk

5. Remove the filters from the hybridization solution, and immediately immerse them in 2 × SSC at room temperature. Agitate the fluid continuously. Replace the fluid every 5 minutes until the amount of radioactivity on the filters remains constant (as measured with a hand-held minimonitor).

6. Adjust the temperature of a circulating water bath to $T_m - 25°C$. Dispense 5 ml of 2 × SSC into each of 20 glass test tubes (17 mm × 100 mm). Monitor the temperature of the fluid in one of the tubes with a thermometer. Incubate the tubes in the water bath until the temperature of the 2 × SSC is $T_m - 25°C$. The 2 × SSC in each of these tubes will be used separately for each temperature increase (see steps 7–10).

7. Transfer the filters individually to four empty glass tubes, separating the filters containing the vector and target DNAs, and add 1 ml of 2 × SSC (from one of the tubes prepared in step 6 and prewarmed to $T_m - 25°C$). Place the tubes in the water bath for 5 minutes.

8. Remove the tubes from the bath, transfer the liquid to scintillation vials, and wash the tubes and filters with 1 ml of 2 × SSC at room temperature. Add the wash solutions to the appropriate scintillation vials.

9. Increase the temperature of the water bath by 3°C, and wait for the temperature of the 2 × SSC in the tubes prepared in step 6 to equilibrate.

10. Add 1 ml of 2 × SSC at the higher temperature to each of the four tubes containing the filters. Place the tubes in the water bath for 5 minutes.

11. Repeat steps 8, 9, and 10 at successively higher temperatures until a temperature of $T_m + 30°C$ is achieved.

12. Place the filters in separate glass tubes (17 mm × 100 mm) containing 1 ml of 2 × SSC, and heat them to boiling for 5 minutes to remove any remaining radioactivity. Cool the solutions in ice, and transfer them to scintillation vials. Wash the filters and tubes used for boiling with 1 ml of 2 × SSC, and add the washing solutions to the appropriate scintillation vials.

13. Use a scintillation counter to measure the radioactivity (by Cerenkov counting, see Appendix E) in all of the vials. Calculate the proportion of the total radioactivity that has eluted at each temperature (i.e., the sum of radioactivity eluted at all temperatures between $T_m - 25°C$ and the temperature at which a given sample was taken divided by the total radioactivity eluted from the filters at all temperatures up to and including 100°C).

    If the experiment has worked well, very little radioactivity should be associated with the filters containing vector DNA alone. Furthermore, this radioactivity should be completely released from the filters at temperatures much lower than the estimated T_m. On the other hand, considerable radioactivity should be associated with the filters containing the target DNA; the elution of this radioactivity should show a sharp temperature dependence. Very little radioactivity should be released from the filters until a critical temperature is reached, and then approximately 90% of the radioactivity should be released during the succeeding 6–9°C rise in temperature. The temperature at which 50% of the radioactivity has eluted from the filters containing the target sequences is defined as the T_i of the hybrid between the probe and its target sequence.

Notes

i. Although the above protocol calls for the use of sodium salts in the solvent used for hybridization, other solutes such as tetramethylammonium chloride or tetraethylammonium chloride can be substituted if desired to determine the T_i in these solvents.

ii. This method can easily be adapted to study the behavior of hybrids formed between probes and target sequences that do not match each other perfectly (Jacobs et al. 1988).

iii. Before synthesizing the probe, check for potential homology and/or complementarity between its sequence and the sequence of the vector used to propagate the target. Most of the commercially available programs to analyze DNA can be used to search commonly used vectors for sequences that match the sequence of the probe closely enough to cause problems during hybridization.

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